HHS Public Access

Author manuscript

J Vis Exp. Author manuscript; available in PMC 2020 June 17.

Isolation of Macrophage Subsets and Stromal Cells from Human and Mouse Myocardial Specimens

Geetika Bajpai[1], **Kory J. Lavine**[1,2,3]

[1]Department of Medicine, Washington University School of Medicine

[2]Department of Immunology and Pathology, Washington University School of Medicine

[3]Department of Developmental Biology, Washington University School of Medicine

Abstract

Macrophages represent the most heterogeneous and abundant immune cell populations in the heart and are central in driving inflammation and reparative responses after cardiac injury. How various subsets of macrophages orchestrate the immune responses after cardiac injury is an active area of research. Presented here is a simple protocol that our lab performs routinely, for the extraction of macrophages from mouse and human myocardium specimens obtained from healthy and diseased individuals. Briefly, this protocol involves enzymatic digestion of cardiac tissue to generate a single cell suspension, followed by antibody staining, and flow cytometry. This technique is suitable for functional assays performed on sorted cells as well as bulk and single cell RNA sequencing. A major advantage of this protocol is its simplicity, minimal day to day variation and wide applicability allowing investigation of macrophage heterogeneity across various mouse models and human disease entities.

Keywords

Immunology and Infection; Issue 154; macrophages; human heart; flow cytometry; heart failure; stromal cells; mouse heart

Introduction

Macrophages represent the most abundant immune cell type in the heart, and they play significant roles in generating robust inflammatory and reparative responses following cardiac injury[1,2,3,4]. Previously, our group identified two major subsets of macrophages in the murine heart derived from distinct developmental origins[5,6]. Broadly, distinct populations of tissue resident cardiac macrophage subsets can be identified based on the cell surface expression of CCR2 (C-C motif chemokine receptor 2). $CCR2^{-}$ macrophages (cell surface expression: $CCR2^{-}MHCII^{low}$ and $CCR2^{-}MHCII^{high}$) are of embryonic origin

Correspondence to: Kory J. Lavine at klavine@wustl.edu.

Disclosures
Authors have nothing to disclose.

Video Link
The video component of this article can be found at https://www.jove.com/video/60015/

(primitive and erythromyeloid lineages), able to self-renew, and represent a dominant population under homeostatic conditions. Resident CCR2^{+} macrophages are of definitive hematopoietic origin, are maintained through recruitment from circulating monocytes, and represent a minor population under homeostatic conditions. Functionally, CCR2^{-} macrophages generate minimal inflammation and are critical for coronary development neonatal heart regeneration[5,7]. In contrast, CCR2^{+} macrophages initiate robust inflammatory responses following cardiac insults and contribute to collateral cardiomyocyte injury, adverse remodeling of the left ventricle, and heart failure progression[8,9].

Recently, we have shown that the human myocardium also contains two distinct subsets of macrophages identified similarly as either CCR2^{-} or CCR2^{+}[8]. Gene expression and functional analyses revealed that human CCR2^{-} and CCR2^{+} macrophages represent functionally divergent subsets and are functionally analogous to CCR2^{-} and CCR2^{+} macrophages found in the mouse heart. Human CCR2^{-} macrophages express robust levels of growth factors, including IGF1, PDGF, Cyr61, and HB-EGF. CCR2^{+} macrophages are enriched in chemokines and cytokines that promote inflammation, such as IL-1b, IL-6, CCL-2, CCL-7, and TNF-a. Stimulated CCR2^{+} macrophages secrete markedly higher levels of the inflammatory cytokine interleukin-1β (IL-1β) in culture. How these subsets differentially contribute to tissue repair and left ventricular (LV) remodeling in the context of cardiac injury remains an area of active research.

Flow-cytometry based analysis of macrophage heterogeneity in the mouse and human heart requires digesting the cardiac tissue and generating a single cell suspension followed by flow cytometric analysis or cell sorting for further downstream processes such as bulk RNA sequencing/single cell RNA sequencing or culturing the cells for functional assays. The original protocol for making a single cell suspension from murine hearts were first reported by Nahrendorf group in Nahrendorf et al. 2007[10]. Our lab has adapted and modified the protocol to extract macrophages from the human myocardium. Using the same protocol but with slight modification in staining and gating scheme, CD45^{-} stromal cells from the human myocardium can also be harvested. Presented here, in text and video, is a protocol that is performed routinely for the extraction of macrophages or stromal from the human myocardium.

Cardiac tissue specimens are obtained from adult patients with dilated cardiomyopathy (DCM: idiopathic or familial) or ischemic cardiomyopathy (ICM) undergoing left ventricular assist device (LVAD) implantation or cardiac transplantation. Explanted hearts or LVAD cores are intravascularly perfused with cold saline prior to starting the digestion procedure. It is important to note that “quality” of tissue specimen determined in terms of degree of scarring or adipose tissue infiltration can greatly affect the yield of macrophages. Heart specimens with large areas of scarring will have much lower cell yield and can pose serious technical limitation when desired downstream analysis methods require in vitro cell culturing.

Protocol

The protocol presented has been approved by the Washington University in St. Louis Institutional Review Board (#201305086). All subjects provide informed consent before sample collection and the experiments are performed in accordance with the approved study protocol. The presented protocol is performed with the approval of the Institutional Animal Care and Use Committee at Washington University School of Medicine and follows the guidelines described in the NIH Guide for the Care and Use of Laboratory Animals.

1. Preparation of Human Cardiac Tissue Specimens

1. Flush explanted hearts by cannulating the left and right coronary artery ostia and perfuse with 200 mL of cold saline.
2. Flush LVAD apical cored tissues by cannulating an epicardial vessel and perfuse with 50 mL of cold saline.
3. Dissect tissue specimen from the apical or lateral wall of the left ventricle in cold saline or HBSS using a sterile scissor.
4. Carefully dissect out epicardial fat and chordae tendineae from the specimen using fine scissors.
5. Dissect tissue chunks into pieces weighing approximately 200 mg with help of a sterile blade or scissors.

2. Preparation of Mouse Heart

1. Euthanize the mouse by CO_2 asphyxiation or cervical dislocation.
2. Open the chest cavity with the help of sharp scissors. Use blunt hemostats to lift the heart upwards. Perfuse the heart with cold PBS using a 25 G needle attached to a 5 mL syringe. Perfuse until heart appears blanched in color.
3. Remove the heart and place in a sterile Petri dish on ice.

3. Preparation of Single Cell Suspension

1. Place human cardiac tissue chunks (~200 mg) or murine heart in a sterile Petri dish. Finely mince the tissue using a sterile blade or scissors.
2. Set up the digestions:
    1. Use a final digestion volume of 3 mL per human cardiac tissue chunk (200 mg) or per one murine heart. Final enzyme concentrations are as follows: Collagenase1 (450 U/mL), DNase1 (60 U/mL), Hyaluronidase (60 U/mL).
    2. For each digestion reaction add DMEM and all enzymes to a 15 mL conical tube. With the help of clean forceps, place the finely minced tissue into each reaction tube. Mix well by gentle vortexing.

3. Digest for 1 h at 37 °C in a shaking incubator set to a low to medium agitation speed.

4. After 1 h of digestion, take the tubes out from the incubator and place on ice. Set up 50 mL conical tubes with a 40 μm cell strainer on top. Wet the filters with 2 mL of enzyme deactivating (ED) buffer.

5. Deactivate the digestion enzymes by adding 8 mL of ED buffer to each digestion tube. Then pour the resulting 13 mL total mixture through the 40 μm cell strainer into the 50 mL conical tubes. Transfer the samples back in fresh 15 mL conical tube. This enables optimal cell pelleting and minimizes cell loss during centrifugation.

6. Spin the samples at 400 x *g* for 6 min (Centrifuge set at 4 °C). Discard the supernatant leaving 0.5 mL of media. Resuspend the cell pellet by gentle pipetting and add 1 mL of ACK lysis buffer. Gently swirl the tube and incubate at room temperature for 5 min to perform red blood cell (RBC) lysis.

7. After 5 min in ACK buffer, add 9 mL of DMEM to the sample. Put the lid back on to the tubes and gently invert the tubes to mix, and filter through a 40 μm cell strainer. Collect the filtrate in 15 mL conical tubes.

8. Centrifuge the tubes at 400 x *g* for 6 min and discard the supernatant.

9. Add 1 mL of FACS buffer and resuspend the pellet. Then transfer in the cells in FACS buffer to a 1.5 mL microcentrifuge tube. Centrifuge again at 400 x *g* for 5 min. Discard the supernatant and resuspend the pellet in 100 μL of FACS Buffer. A single cell suspension is now ready for antibody staining.

4. Antibody Staining

1. A typical human antibody panel consists of the following antibodies: CD45-PercpCy5.5, CD14-PE, CD64-FITC, HLA-DR-APC/Cy7, CCR2-APC. Please refer to Table 1. Add all antibodies to the heart samples at 1:50 dilution and incubate for ~30–40 min at 4 °C in the dark. Proceed to step 4.4 below.

2. For stromal cells (endothelial cells, fibroblast, smooth muscle cells), add DRAQ5 (1 μM final concentration) and CD45-percpCy5.5. Please refer to Table 1. Incubate for 30 min at 4 °C in the dark. Proceed to step 4.4 below.

3. For murine heart macrophages add the following antibodies: CD45-PercpCy5.5, CD64-APC, MHCII-APC/Cy7, CCR2-BV421, and Ly6GFITC. Please refer to Table 2. Add all antibodies to the heart samples at 1:100 dilution and incubate for ~30–40 min at 4 °C in the dark. Proceed to step 4.4 below.

4. Wash the samples twice in FACS buffer. For each wash, add 1 mL of FACS buffer, gently vortex, and centrifuge at 400 x *g* for 5 min, resuspend in 350 μL of FACS buffer and add DAPI (1 μM, final concentration). Samples are now ready for FACS analysis/sorting.

Representative Results

The protocol described allows isolation of macrophages from mouse and human myocardium. Using the same protocol, but with a different staining and gating strategy, stromal cells can also be harvested from the human myocardium. FACS results presented here were acquired either on BD LSRII or BD FACS ARIA III platform. Compensation controls were generated from single color control samples from stained splenocytes. Figure 1 shows unprocessed and processed human LVAD core. Figure 2 shows the gating scheme for the flow sorting of $CCR2^{-}$ and $CCR2^{+}$ human macrophages. Figure 3A shows the gating scheme for $CD45^{-}$ stromal cells from human myocardium and Figure 3B shows images of Wright stained FACS sorted $CD45^{+}$ and $CD45^{-}$ cells. Figure 4 describes the gating scheme to sort macrophages from a mouse heart.

Discussion

The protocol allows for the extraction of various macrophage subsets from human myocardium. The protocol is simple and takes 3 to 4 hours to prepare single cell suspension ready for FACS analysis. Although the protocol is relatively simple to perform, there are certain technical aspects that need to be considered which will minimize variability. Firstly, working in timely fashion with human tissue is necessary for optimal cell viability. It is important to keep the tissue in cold saline/HBSS to minimize cell death. It is also necessary to remove epicardial fat and other connective tissue from the myocardial specimen. Consistent tissue mincing and digestion times will reduce sample to sample variation.

There is both intra-assay and inter-assay variability in tissue digestions and subsequent cell yields. This is one of the limitations in preparing a single cell suspension from tissues. The most important way to minimize this is by making sure enzymes are relatively new, properly aliquoted, and stored at −80 °C. Aliquots should be used one time only and should not be saved or frozen again. Enzymes used are sensitive to freeze thaw cycles. Another consideration is temperature of digestion and shaking speed. Using a thermostat-controlled shaker that evenly distributes heat helps to minimize digestion variability and improve cell viability.

It is important to mention that absolute yield of macrophages from human myocardium is generally not very high. Usually the cell yield varies from ~20,000 to 50,000 total macrophages per 1,200–1,500 mg of tissue. This becomes challenging when the desired downstream method of analysis involves cell culture assays. Phagocytosis, chemokine/cytokine production, cell stimulation, morphometry, and gene expression analyses (microarray, bulk RNA sequencing, and single cell RNA sequencing) can be easily performed. The quality of the tissue also determines the ease of digestion and subsequent cell yield. If the tissue is fibrous and scarred, digestion efficiency is suboptimal, and macrophage yield is likely to be very low.

Another aspect to consider is that myocardial tissue digestion leads to significant debris formation. This causes the pellet to appear loose. Thus, one must be careful to not discard the supernatant during wash steps by simple decanting. Using a suction/vacuum waste

collection flask with a Pasteur pipette to collect the supernatant is advisable. Also, maintaining the centrifuge at 4 °C will help minimize the cell death and sample loss.

Although this protocol describes a way to extract macrophages from human myocardial tissue samples, successful isolation of macrophage subsets can also be achieved from mouse hearts without significant changes or modifications.

Acknowledgments

This project was made possible by funding provided from the Children's Discovery Institute of Washington University and St. Louis Children's Hospital (CH-II-2015-462, CH-II-2017-628), Foundation of Barnes-Jewish Hospital (8038-88), and the NHLBI (R01 HL138466, R01 HL139714). K.J.L. is supported by NIH K08 HL123519 and Burroughs Welcome Fund (1014782).

References

1. Pinto AR et al. An abundant tissue macrophage population in the adult murine heart with a distinct alternatively-activated macrophage profile. PLoS One. 7 (5), e36814 (2012). [PubMed: 22590615]
2. Hulsmans M et al. Macrophages facilitate electrical conduction in the heart. Cell. 169 (3), 510–522.e20 (2017). [PubMed: 28431249]
3. Hulsmans M et al. Cardiac macrophages promote diastolic dysfunction. Journal of Experimental Medicine. 215 (2), 423–440 (2018). [PubMed: 29339450]
4. Aurora AB et al. Macrophages are required for neonatal heart regeneration. Journal of Clinical Investigation. 124 (3), 1382–1392 (2014). [PubMed: 24569380]
5. Lavine KJ et al. Distinct macrophage lineages contribute to disparate patterns of cardiac recovery and remodeling in the neonatal and adult heart. Proceedings of the National Academy of Sciences of the United States of America. 111 (45), 16029–16034 (2014). [PubMed: 25349429]
6. Epelman S et al. Embryonic and adult-derived resident cardiac macrophages are maintained through distinct mechanisms at steady state and during inflammation. Immunity. 40 (1), 91–104 (2014). [PubMed: 24439267]
7. Leid J et al. Primitive embryonic macrophages are required for coronary development and maturation. Circulation Research. 118 (10), 1498–1511 (2016). [PubMed: 27009605]
8. Bajpai G et al. The human heart contains distinct macrophage subsets with divergent origins and functions. Nature Medicine. 24 (8), 1234–1245 (2018).
9. Dick SA et al. Self-renewing resident cardiac macrophages limit adverse remodeling following myocardial infarction. Nature Immunology. 20, 29–39 (2019). [PubMed: 30538339]
10. Nahrendorf M et al. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. Journal of Experimental Medicine. 204 (12), 3037–3047 (2007). [PubMed: 18025128]

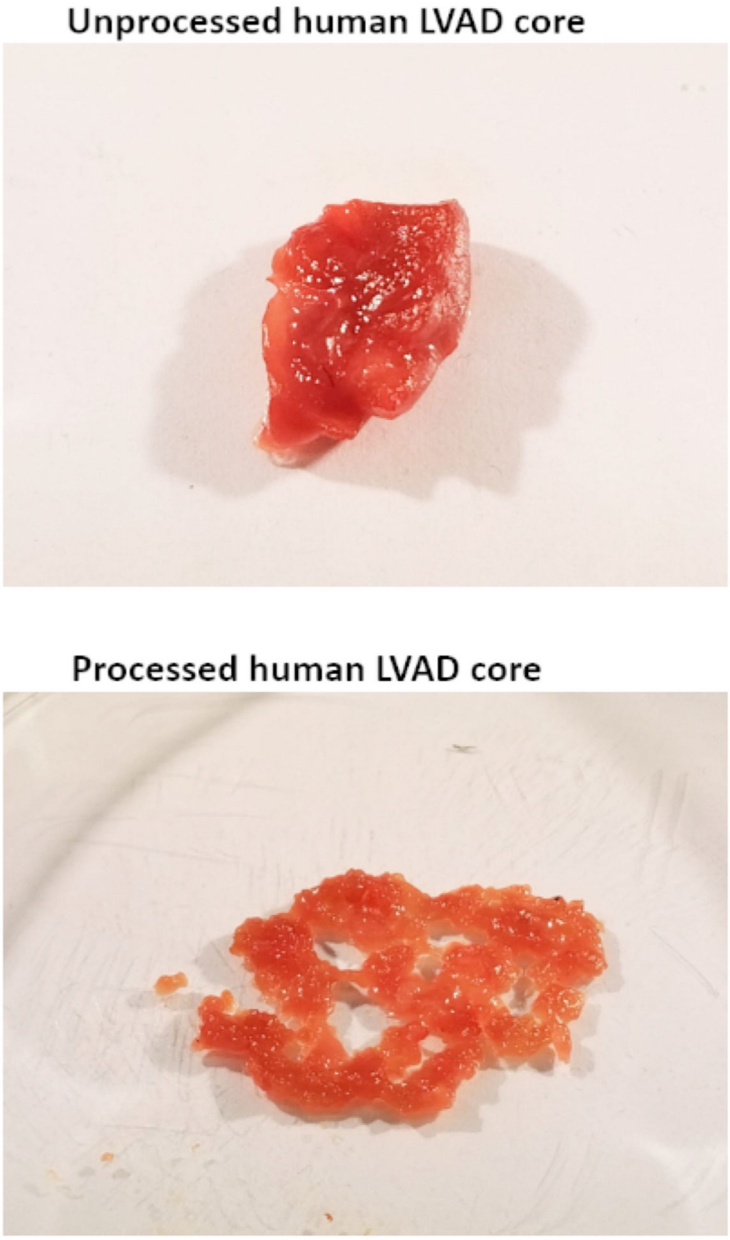

Figure 1:
The human LVAD tissue core before and after processing.

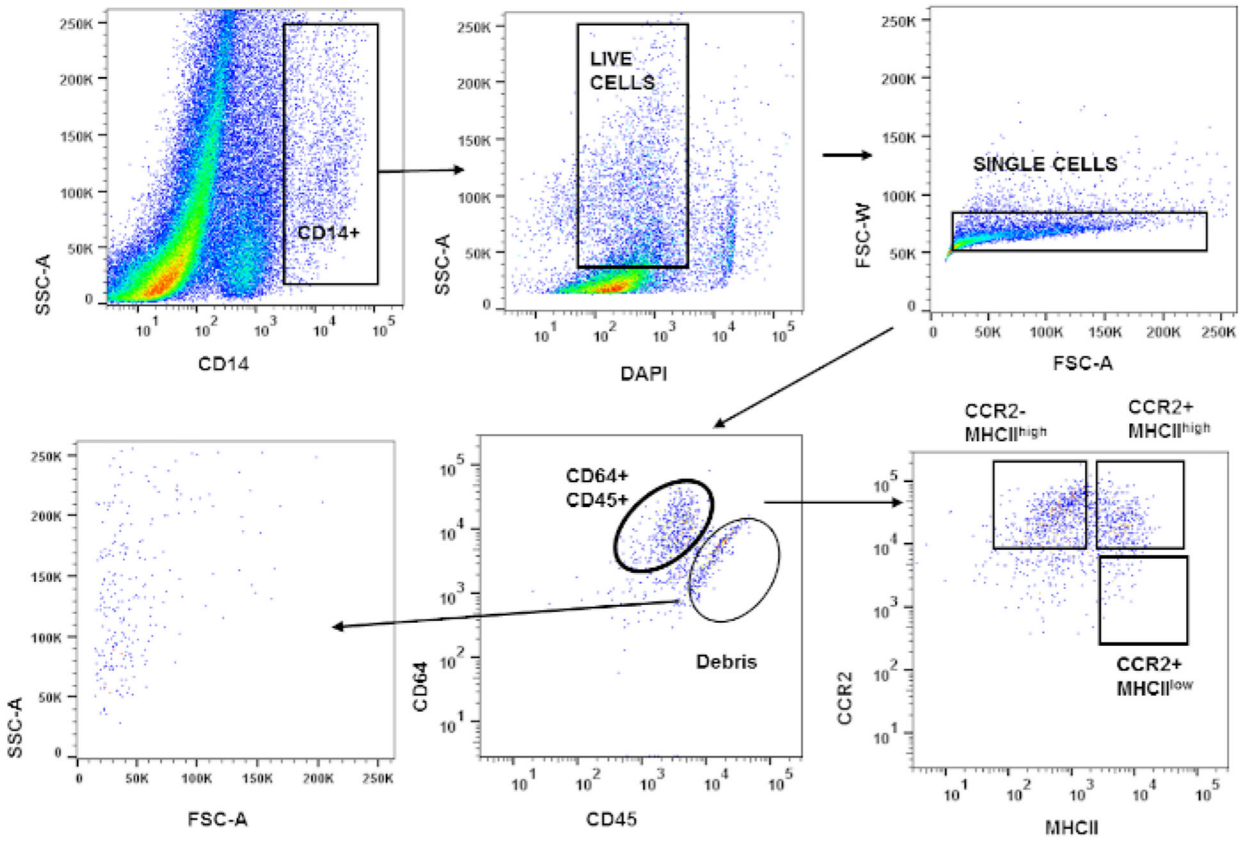

Figure 2:

Flow cytometry gating scheme utilized to identify and characterize cardiac macrophage populations in dilated cardiomyopathy (DCM) or ischemic cardiomyopathy (ICM) specimens.

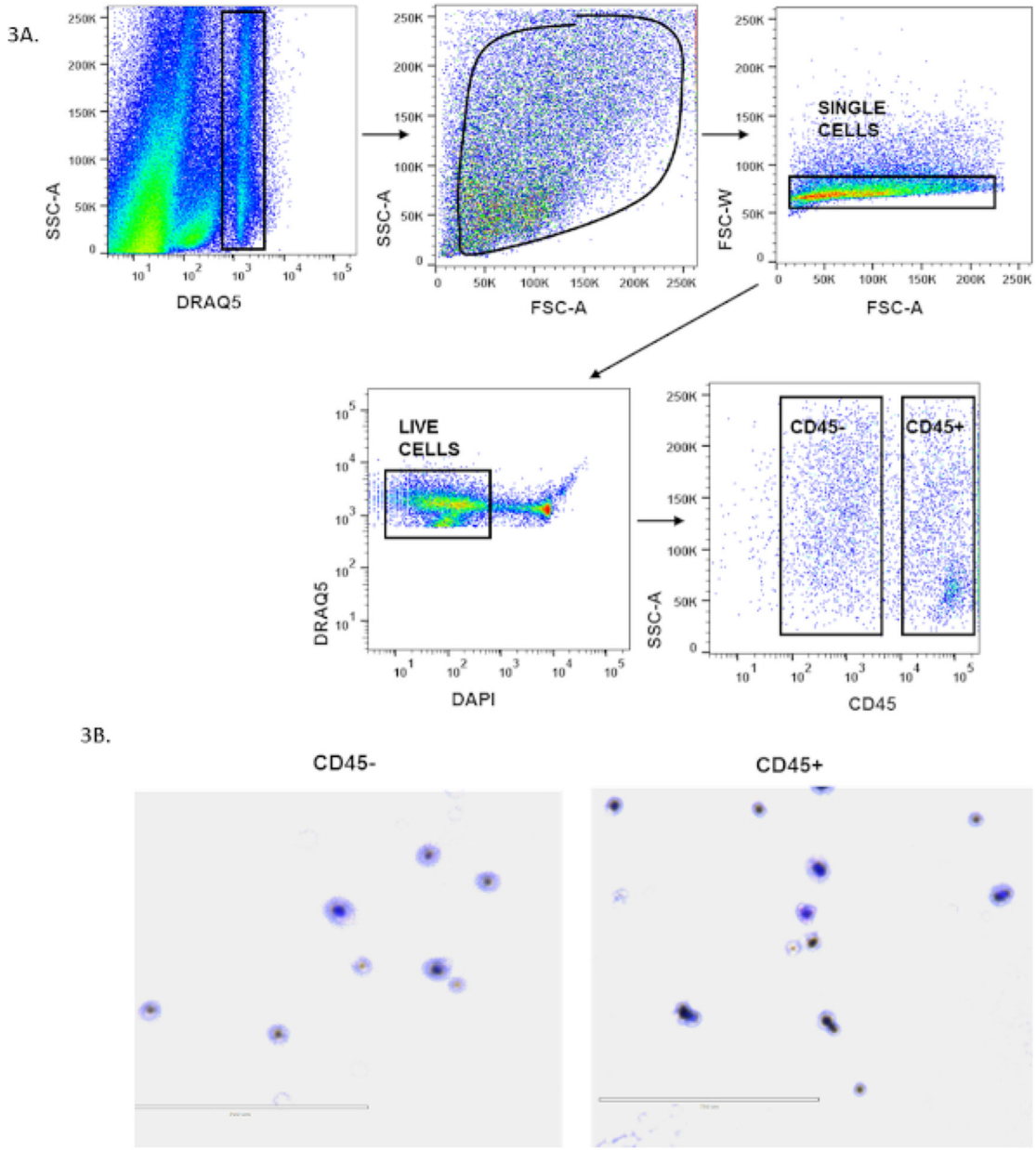

Figure 3: Flow cytometry gating scheme to isolate CD45^{-} and CD45^{+} stromal cells from human samples.

(**A**) Flow cytometry gating scheme utilized to isolate CD45^{-} stromal cells from human ischemic cardiomyopathy (ICM) or dilated cardiomyopathy (DCM) specimens. (**B**) Wright stained FACS sorted CD45^{-} and CD45^{+} cells. Scale bars = 100 μm.

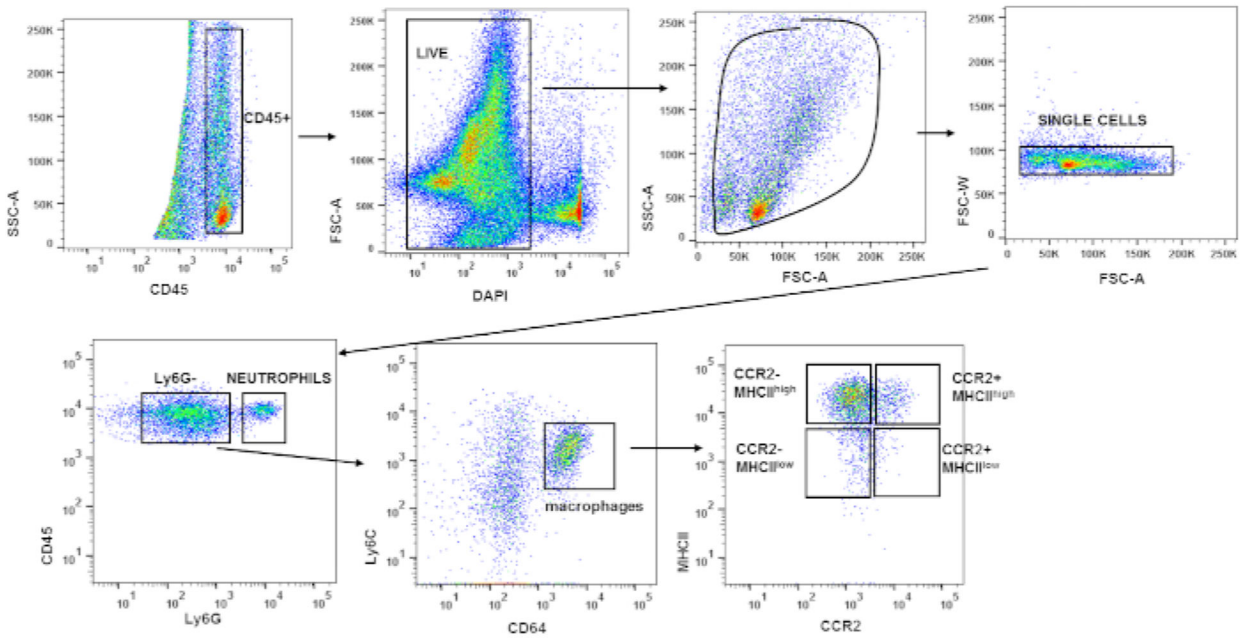

Figure 4:

Flow cytometry gating scheme to isolate various macrophage subsets from the mouse heart.

Table 1:

Antibody panel for human myocardium specimen.

Antigen	Fluorophore	Clone	Manufacturer
CD45	PercpCy5.5	2D1	Biolegend
CD64	FITC	10.1	Biolegend
CD14	PE	M5E2	Biolegend
CCR2	APC or BV421	K036C2	Biolegend
MHCII	APC/Cy7	L243	Biolegend
DRAQ5			Thermo Fisher
DAPI			Thermo Fisher

Table 2:

Antibody panel for mouse heart specimen.

Antigen	Fluorophore	Clone	Manufacturer
CD45	PercpCy5.5	30-F11	Biolegend
CD64	APC	X54–5/7.1	Biolegend
Ly6G	PE/Cy7	1A8	Biolegend
Ly6C	FITC	HK1.4	Biolegend
CCR2	BV421	SA203G11	Biolegend
MHCII	APC/Cy7	M5/114.15.2	Biolegend
DRAQ5			Thermo Fisher
DAPI			Thermo Fisher